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APPLICATION OF AN IMPROVED METHOD OF TWO-DIMENSIONAL ELECTROPHORESIS TO THE SYSTEMATIC STUDY OF HORSESHOE CRABS

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Abstract

Protein constituents of skeletal and cardiac muscles of extant horseshoe crabs, *Tachypleus tridentatus, T. gigas, Carcinoscorpius rotundicauda,* and *Limulus polyphemus,* were examined by two-dimensional gel electrophoresis in order to investigate the applicability of this technique in systematics and to obtain more data on phylogenetic relationships of the four species. Electrophoretic patterns were analyzed by both comparison of spot positions only and that of presumed protein groups (positions and shapes).

Our phenetic analyses confirmed the disparate position of the North American species, *L. polyphemus,* and the similarity of the three Asian species. Our cladistic analysis suggested a sister-group relationship of *T. tridentatus* and *C. rotundicauda.* This suggestion, however, was based on a small number of characters.

On the basis of this electrophoretic study, we suggest that conventional native (non-denaturing) gel electrophoresis for enzymes is optimal for measuring genetic differences among organisms at lower taxonomic levels, but two-dimensional gel electrophoresis is best for overall protein comparison.

Introduction

There are four currently recognized extant species of horseshoe crabs, *Tachypleus tridentatus, T. gigas, Carcinoscorpius rotundicauda,* and *Limulus polyphemus. T. hoeveni,* described by Pocock (1902), was considered by Waterman (1958) to be a synonym of *T. gigas.*

Previous phylogenetic studies on those four species have utilized external morphology, hybridization tests, and amino acid sequencing (Pocock, 1902; Sekiguchi and Sugita, 1980; Shishikura *et al.,* 1982; Srimal *et al.,* 1985). These studies have shown that *Limulus polyphemus,* which inhabits the east coast of North America, is disparate from the three southeast Asian species.

The classical or morphological view places two of these Asian species in the genus *Tachypleus* and considers *Carcinoscorpius* to be a monotypic genus (Pocock, 1902; Størmer, 1952). Contradictory biochemical, embryological, and genetical data, however, suggest that *T. tridentatus* is more similar to *C. rotundicauda* than to *T. gigas* (Sekiguchi and Sugita, 1980; Shishikura *et al.,* 1982; Srimal *et al.,* 1985).

Since previous biochemical studies on horseshoe crabs have dealt only with two proteins, coagulogen and hemocyanin (Shishikura and Sekiguchi, 1978; Sugita and

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Sekiguchi, 1981; Shishikura *et al.,* 1982; Srimal *et al.,* 1985), the number of protein characters examined has been too small for reasonably deducing phylogenetic relationships. Because two-dimensional gel electrophoresis can detect more subtle protein differences than conventional electrophoresis, it can provide information on many kinds of proteins. However, the usefulness of the data for phylogenetic analyses has not yet been examined (Wiley, 1981).

In this study, proteins from cardiac and skeletal muscles of the four species of horseshoe crabs were separated by two-dimensional gel electrophoresis (1) to examine its applicability to phylogenetic analysis, (2) to obtain new data on phylogenetic relationships among the three Asian species, and (3) to re-examine the disparate taxonomic position of the North American species. We also compared the applicability of the two-dimensional gel electrophoresis method with that of the conventional method which utilizes a native (non-denaturing) gel. Previous studies have suggested that the two-dimensional method gives a smaller magnitude of both intraspecific and interspecific differences than does the latter method (Brown and Langley, 1979; McConkey *et al.,* 1979; Walton *et al.,* 1979; Racine and Langley, 1980; Aquadro and Avise, 1981; McConkey, 1982).

Materials and Methods

Tachypleus tridentatus was collected in Imari, Japan. *Tachypleus gigas* and *Carcinoscorpius rotundicauda* were obtained from Bangsaen, Thailand. *Limulus polyphemus* was purchased from the Marine Biological Laboratory, Woods Hole, Massachusetts, USA.

The tubular heart (cardiac muscle) and a pair of skeletal muscles that connect the prosoma with gills were dissected from each specimen and cleaned quickly in filtered seawater to prevent contamination from other tissues and hemolymph. The heart was cut into anterior, central, and posterior parts for comparison of protein components in respective sections. The heart parts and skeletal muscles were cut into small pieces and stored at −80°C.

Two-dimensional electrophoresis was carried out essentially according to the method improved by Hirabayashi (1981). One piece (0.1 g) of each muscle was homogenized with a Dounce homogenizer in 4 ml of an extraction medium of 8 *M* urea, 1 *M* KI, 0.1 m*M* N^{α}-tosyl-L-lysylchloromethanehydrochloride, 10% β-mercaptoethanol, and 0.1 *M* Tris-HCl, pH 7.5. This homogenate was dialyzed against 7 *M* urea for 3–4 hours and centrifuged at 30,000 × *g* for 30 minutes with a Beckman JA-14 rotor. For single species runs, 60 μl and 40 μl of the supernatants from cardiac and skeletal muscles, respectively, were loaded on the first dimension isoelectric focusing gels. When samples from two species were compared, a 1:1 mixture of 80 μl (40 μl from each heart) or 50 μl (25 μl from each skeletal muscle) was also loaded. Isoelectric focusing was carried out at 500 V for 24 hours with cardiac muscle and 30 hours with skeletal muscle. The remaining supernatants and precipitates were each mixed with a hot 1% agarose solution containing 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.02% bromophenol blue, and 0.05 *M* Tris-HCl, pH 6.8. Each mixture was chilled in a glass tube and stored as reference for total supernatant or precipitate components of the second dimension SDS-polyacrylamide gel electrophoresis. The second dimension, with a concentration gradient of acrylamide (12–20%), was carried out at 30 mA, when the bromophenol blue tracking dye was in the stacking gel, and then continued at 60 mA until the dye reached the lower end of the running gel.

For optimal comparison of two-dimensional patterns, we electrophoresed ex-

tracts from each of two species and a mixture of the two at the same time ("triplet method," Fig. 1). A close examination of the mixture pattern (Fig. 1b) was indispensable to confirm overlapping (or sharing) of spots suspected to be similar from individual patterns (Fig. 1a, c). Preliminary examination of cardiac muscle with the "triplet method" did not show significant differences among the three male and one female specimens of *T. tridentatus* and among the anterior, central, and posterior parts of a single heart of the animal. However, we used only the anterior part, since the sample was large enough. For interspecific analyses, we used six "triplet" combinations to compare patterns among the four species. In all, about 100 obvious spots were examined on each pattern of cardiac and skeletal muscles for each of the four species. We only used those spots that could be consistently matched or clearly defined.

The two-dimensional electrophoresis method, applied herein, has two advantages over those used in previous studies. First, the substitution of agarose for acrylamide in the first dimension gel allowed us to analyze proteins of higher molecular weights (up to about 250 KD). Second, we used four kinds of ampholytes (rather than one or two) in the first dimension for wider pH coverage (pH 3.5–9.5). These changes, plus a concentration gradient of acrylamide in the second dimension, permitted us to analyze about 100 proteins, presenting many more characters for systematic study than other methods of electrophoresis.

Results

Typical two-dimensional electrophoresis patterns of cardiac muscle of the four species are shown in Figures 1 and 2. Ninety-seven of about 100 major spots for each of the four species were "shared" (that is, having overlapping positions) by at least two species. These spots were numbered in the order of their relative molecular weights (Fig. 3). Those shown in boxes below Nos. 5 and 6 (Fig. 2) may have been derived from hemolymph proteins, probably hemocyanin. Hemolymph could not be removed completely, even though the muscle was thoroughly washed in seawater.

The sharing (overlapping) of cardiac muscle spots by the four species is shown in Table I, where the numbers correspond to those in Figure 3. The numbers of spots shared by any two of the three Asian species were almost the same and roughly twice those shared by each Asian species and the North American one (Table II).

When only the spot position was considered, we regarded two similar-appearing spots separately even if both were presumed to be derived from the same kind of proteins by their similar morphology and positions relative to other spots. Nos. 20 and 21, for example, were counted separately for analysis of the position only (Table I), although they may have been counterparts.

Next we considered such spots (*e.g.,* Nos. 20 and 21) as belonging to the same protein group and counted all examined spots as representing protein groups (Table III). In some instances, no spot was shared for the presumed protein group, *e.g.,* Nos. 98–108 in Table III. This resulted in a difference of five shared spots for cardiac muscle between the two approaches (see Tables I and III).

Tabulation of shared cardiac muscle protein spots (Table IV) gave a similar result to that when only the spot position was considered (Table II), the usual method of analyzing two-dimensional electrophoretic patterns. Therefore, both approaches support the view that the three Asian species are more similar to one another than to the North American species.

Cardiac muscle was used in this study both because of its importance to life and the homogeneity of its cell population. For similar reasons, we also examined skeletal

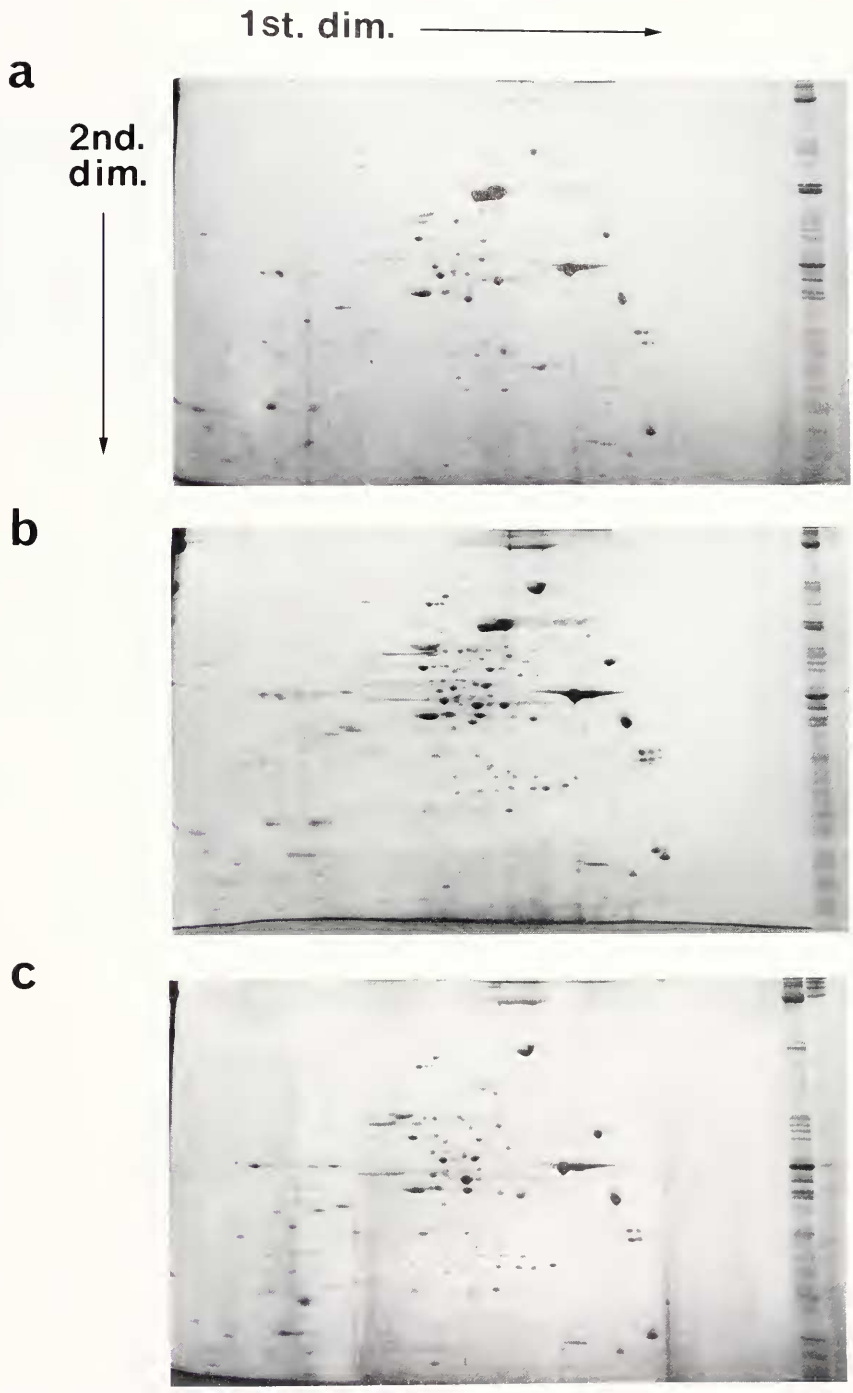

FIGURE 1. The "triplet patterns" of cardiac muscle. For optimal comparison of two-dimensional patterns, we electrophoresed extracts from each species (a, c) and a mixture of the two (b). a, *Tachypleus tridentatus;* c, *Limulus polyphemus.* Two lanes at the right-hand end of each pattern are references for total supernatant (left) and precipitate (right) components.

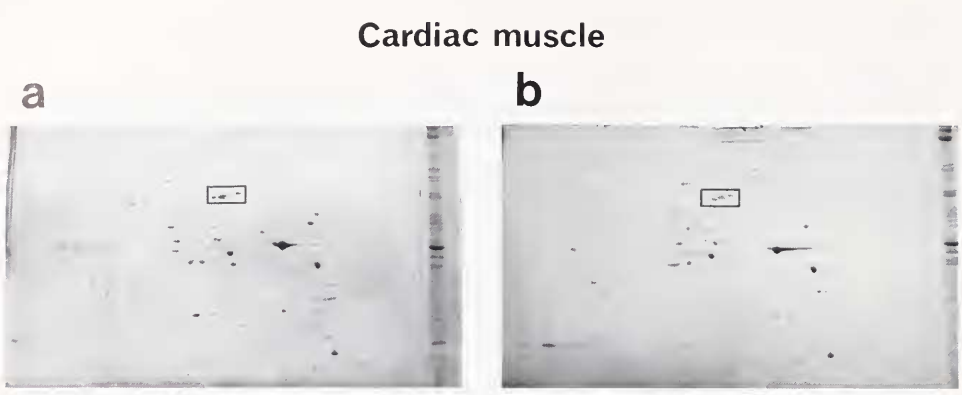

FIGURE 2. The typical two-dimensional electrophoresis patterns of cardiac muscle. a, *Tachypleus gigas;* b, *Carcinoscorpius rotundicauda.* The spots in boxes were probably derived from hemolymph proteins.

muscle that connects the prosoma with the gills. With skeletal muscle, 97 of about 100 major spots on two-dimensional electrophoresis patterns (Fig. 4) were recognized as shared (*i.e.,* having overlapping positions) between at least two species, and shared spots by each pair of species were counted as before (see also Table II). Re-examination for shared spots in protein groups (*i.e.,* similar spot morphology and positions) gave a similar result (Table IV), although it differed by 22 spots. Skeletal muscle patterns also confirm the greater similarity among the Asian species when compared to the North American species.

Using the results in Table IV, we quantified the similarity among the four species

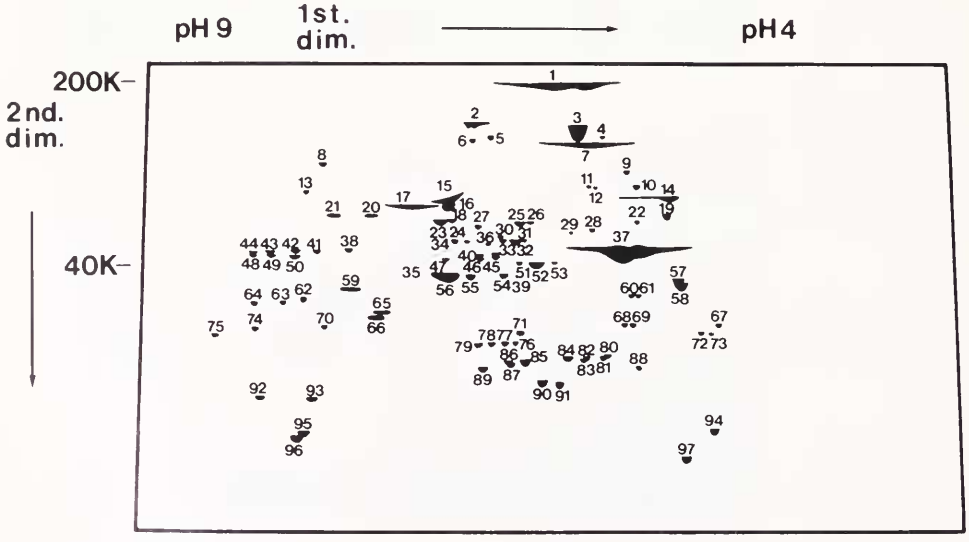

FIGURE 3. The schematic drawing of 97 spots of cardiac muscle. These spots, numbered in the order of their relative molecular weights, were shared by at least two of the four species.

TABLE I

Sharing of spots (positions) for cardiac muscle

Species	No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
T. tridentatus		+	+	+		+	+	+	+	+	+	+	+	+	+	+		+	+		+		+	+	+	+
T. gigas		+		+	+	+	+	+	+	+	+			+	+		+	+	+	+		+	+	+	+	+
C. rotundicauda		+	+	+	+	+	+	+		+	+	+	+			+	+	+	+	+	+			+	+	
L. polyphemus		+		+		+		+		+	+							+	+			+		+	+	+

Species	No.	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
T. tridentatus		+	+			+	+	+	+	+	+		+		+	+	+	+	+		+	+		+	+	+
T. gigas			+	+	+		+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
C. rotundicauda			+	+	+		+	+		+	+	+	+	+	+		+	+	+		+	+	+	+	+	+
L. polyphemus		+	+			+		+	+			+	+	+		+		+	+	+						

Species	No.	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75
T. tridentatus		+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	+		+	+	+			+	+
T. gigas		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+
C. rotundicauda		+			+		+	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	
L. polyphemus					+		+	+	+								+	+								

Species	No.	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97
T. tridentatus		+	+	+	+		+	+	+	+	+	+		+	+	+		+	+	+	+	+	+
T. gigas		+	+	+	+	+		+		+			+	+			+	+	+	+		+	
C. rotundicauda		+	+	+			+	+	+	+	+	+		+	+		+	+	+	+	+		
L. polyphemus						+	+		+	+	+	+	+			+			+		+		+

The symbol (+) represents the presence of a spot shared by at least two of the four species on the electrophoresis pattern of the species.

TABLE II

Number of shared spots

Combination	Cardiac muscle	Skeletal muscle
T. tridentatus and *T. gigas*	62	71
T. tridentatus and *C. rotundicauda*	62	68
T. gigas and *C. rotundicauda*	63	65
T. tridentatus and *L. polyphemus*	36	46
T. gigas and *L. polyphemus*	31	47
C. rotundicauda and *L. polyphemus*	30	45

by applying the formula: $F = 2N_{xy}/(N_x + N_y)$ after Aquadro and Avise (1981). In this formula, F is the similarity between species x and y, N_{xy} is the number of spots shared by x and y, and N_x and N_y are the numbers of spots scored for x and y, respectively. These calculations, shown in Table V, revealed that the similarity between any two of the three Asian species approximated 0.6 for both skeletal and cardiac muscles. Between the North American species and each Asian species, the similarity for cardiac and skeletal muscles was about 0.35 and 0.45, respectively.

If electrophoretic data, when analyzed phenetically, refutes the classical phylogenetic hypothesis, the data should be carefully examined for possible false similarities due to retention of ancestral character states (Baverstock *et al.*, 1979). Both Farris (1974) and Baverstock *et al.* (1979) showed that cladistic analysis of electrophoretic data can give phylogenetic relationships that differ from those inferred from the data analyzed phenetically.

In general, electrophoretic data is analyzed phenetically using the similarity-clustering method. However, phylogenetic relationships, when based only on the similarity, make the unverifiable assumption that evolutionary rates of organisms are uniform. If evolutionary rates are not uniform, two differentiated species, which have evolved at relatively slow rates, may show many shared characters (*i.e.*, spots on electrophoresis patterns) due to retention of ancestral character states. At the same time, one of two closely related species, which has evolved relatively fast, may show, as derived character states, many unshared characters (spots). This may lead to the false conclusion that the two closely related species (of the three) are remote and the two differentiated species are similar. It is therefore important to subject electrophoretic data to cladistic analysis without assuming that evolutionary rates are uniform. However, to identify the ancestral or derived character state for each group of proteins is difficult. As pointed out by Baverstock *et al.* (1979), only the outgroup criterion is suitable for recognizing the character states with electrophoretic data.

Accepting their opinion and using the principle that derived character states held in common imply phylogenetic relationships, we analyzed our electrophoretic data for both cardiac (Table III) and skeletal muscles cladistically by regarding *Limulus polyphemus* as the outgroup, since no previous study has contradicted its disparate position relative to the three other species. The results of our cladistic analysis showed a sister-group relationship between *Tachypleus tridentatus* and *Carcinoscorpius rotundicauda* among the three Asian species. However, this conclusion is based on 1 of the 42 protein groups examined for cardiac muscle (Nos. 20–21 in Table III) and on 3 of 51 for skeletal muscle, since these were the only ones where we could regard overlapping spots as derived character states held in common.

Comparison of spot positions only is less complicated and more suitable for phe-

TABLE III

Sharing of spots in presumed protein groups for cardiac muscle

Species	No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
T. tridentatus		+	+	+	○	+	+	+	+	+	+	+	+	+	+	+	○	+	+	○		+		+	+	+	+	+	+
T. gigas		+	○	+	+	+	+	+	+	+	+		○	+	+	○	+	+	+	+		*		+	+	+	○	○	+
C. rotundicauda		+	+	+	+	+	+	+		+	+	+	+		○	+	+	+	+	+		+	○	+	+	+	△	△	+
L. polyphemus		+	△	+	△	+	○	+		+	+				△	△	△	+	+	△		*		+	+	+	+	+	+

Species	No.	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54
T. tridentatus		○		+	+	+	+	+	+	○	+		+	+	+	+	+		+	+		+	+	+	+	+	+	+
T. gigas		+	+	○	+	+	○	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	+
C. rotundicauda		+	+	△	+	+	△	+	+	+	+	+	+		+	+	+		+	+	+	+	+	+	○	○	○	+
L. polyphemus		△		+	○	+	+	○	○	+	+	+	○	+	○	+	+	+		○			○		△	△	△	+

Species	No.	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81
T. tridentatus		+	+	+	+	+	+	+	+		+	+	+	+	○	○	+	+	○	○	+	+	+	+	+	+		+
T. gigas		+	+	+	+	+	+	+	+	+	+	+	+	○	+	+	+	+	+	+	+	+	+	+	+	+	+	
C. rotundicauda		○	+	+	+	+	+	+	+	+	+	+	+	△	+	+	+	+	+	+	+	○	+	+	+	○		+
L. polyphemus		△	+	+	+	○	○		○	○			+	+	△	△		+	△	△	○		○	○	○	△	+	+

Species	No.	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108
T. tridentatus		+	+	+	+	+		+	+	+	○	+	+	+	+	+	+	○	○	○	○	○	○	○	○	○	○	○
T. gigas		+		+	○	○	+	+	○		+	+	+	+		+	○	△	△	△	△	△	△	△	△	△		△
C. rotundicauda		+	+	+	+	+		+	+		+	+	+	+	+		△	▽	▽	▽	▽	▽	▽	▽		▽	△	▽
L. polyphemus			+	+	+	+	+	○	△	+	△		+	○	+		+	□	□	□		□	□	□	▽	□	▽	□

The symbols (+) and (*) represent the presence of a spot shared by at least two species on the pattern of the species, that is, they show the presence of a protein electrophoretically common to that of other species in a group of proteins with probably identical character. The symbols (○), (△), (▽), and (□) show the presence of an unshared spot on the pattern of the species. A blank indicates the absence of a spot.

TABLE IV

Number of shared spots in presumed protein groups

Combination	Cardiac muscle	Skeletal muscle
T. tridentatus and *T. gigas*	60	62
T. tridentatus and *C. rotundicauda*	60	57
T. gigas and *C. rotundicauda*	62	60
T. tridentatus and *L. polyphemus*	36	41
T. gigas and *L. polyphemus*	30	43
C. rotundicauda and *L. polyphemus*	30	39

netic analysis than use of protein groups (positions and shapes). However, the latter can be used for both cladistic and phenetic analyses.

DISCUSSION

Our phenetic analyses support the view that *Limulus polyphemus* is disparate from the three Asian species, as previously reported (Pocock, 1902; Shuster, 1962; Shishikura and Sekiguchi, 1978; Sekiguchi and Sugita, 1980; Dorai, *et al.,* 1981; Sugita and Sekiguchi, 1981; Shishikura *et al.,* 1982; Yamamichi and Sekiguchi, 1982; Srimal *et al.,* 1985). These analyses also show that the three Asian species are equally

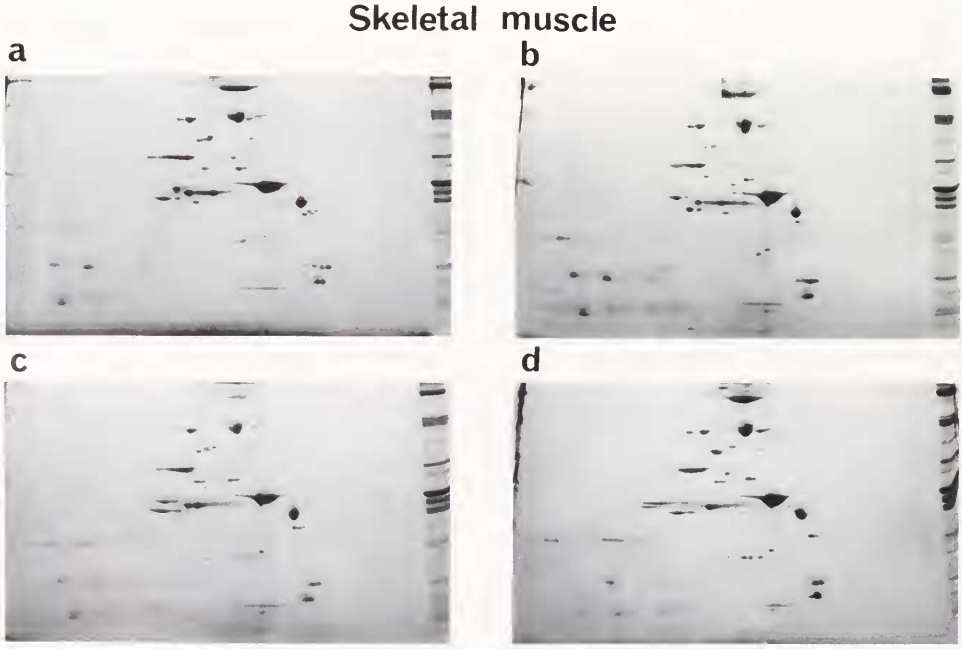

FIGURE 4. The typical two-dimensional electrophoresis patterns of skeletal muscle. a, *Tachypleus tridentatus;* b, *Tachypleus gigas;* c, *Carcinoscorpius rotundicauda;* d, *Limulus polyphemus.* The reference for total supernatant components is shown on the right-hand end.

TABLE V

Similarity among the four species based on presumed protein groups

Combination	Cardiac muscle			Skeletal muscle		
	N_x	N_y	F	N_x	N_y	F
T. tridentatus and *T. gigas*	100	100	0.60	95	97	0.65
T. tridentatus and *C. rotundicauda*	100	98	0.61	95	99	0.59
T. gigas and *C. rotundicauda*	100	98	0.63	97	99	0.61
T. tridentatus and *L. polyphemus*	100	88	0.38	95	85	0.46
T. gigas and *L. polyphemus*	100	88	0.32	97	85	0.47
C. rotundicauda and *L. polyphemus*	98	88	0.32	99	85	0.42

Calculation of the similarity (F) is according to Aquadro and Avise (1981).

similar to each other. This supports the biochemical, embryological, and genetical evidences which show that differences among the three Asian species are not sufficient to justify division into two genera (Sekiguchi and Sugita, 1980; Shishikura *et al.*, 1982; Srimal *et al.*, 1985). However, our phenetic analyses for cardiac and skeletal muscles do not give new insight into the problem of whether *Tachypleus tridentatus* is more similar to *Carcinoscorpius rotundicauda* than to *Tachypleus gigas.*

On the other hand, our cladistic analysis shows that *T. tridentatus* may be more closely related to *C. rotundicauda* than to *T. gigas,* thus supporting the biochemical, embryological, and genetical view cited above. However, only 4 of the 93 cardiac and skeletal muscle protein groups examined indicate these relationships. This number probably is too small to comfortably deduce phylogenetic relationships. Therefore, we must be careful before drawing a conclusion from our data. Nevertheless, all cases, in which derived character states are shared, suggest a sister-group relationship between *T. tridentatus* and *C. rotundicauda.*

The ambiguity of phylogenetic relationships among the Asian species of horseshoe crabs suggested by our phenetic and cladistic analyses raises doubts about the applicability of two-dimensional electrophoresis for phylogenetic studies. Some concerns for this method have been recognized by McConkey (1982). The most important is that two-dimensional electrophoresis gives information only about isoelectric points and molecular weights of proteins and tells nothing about their functions, even though proteins with similar points and weights may have the same functions. The second major concern is that adjacent or partially overlapping large spots may appear as one spot. This may be addressed by using varing amounts of proteins (*i.e.*, volumes of supernatants) for two-dimensional analysis.

The use of two-dimensional gel electrophoresis in phylogenetic studies has been more controversial than that of conventional (native gel) electrophoresis. Aquadro and Avise (1981) have shown that genetic distances among rodents, revealed by two-dimensional electrophoresis, is about half of those shown by native gel electrophoresis. In their opinion, kinds of proteins detected by the two methods may be different, and it is not clear whether the charge-equivalent substitutions of amino acids can be detected by two-dimensional electrophoresis, since it involves denaturing of proteins.

Studies of genetic heterozygosity with two-dimensional electrophoresis have also shown lower estimates than those by native gel electrophoresis (Brown and Langley, 1979; McConkey *et al.*, 1979; Walton *et al.*, 1979; Racine and Langley, 1980). These authors have pointed out that the native gel method is more sensitive both to confor-

mational and post-translational protein differences, and to variable proteins (*i.e.*, enzymes) than the two-dimensional method. Enzymes also vary, however, including less variable ones that are substrate-specific, are involved in energy metabolism, or utilize intracellular substrates (Gillespie and Kojima, 1968; Johnson, 1973; Cohen *et al.*, 1973). Therefore, even enzymes detected by native gel electrophoresis can be divided into two types (variable or specific). Furthermore, Brown and Langley (1979) have shown that heterozygosity estimates obtained by native gel electrophoresis of specific enzymes are smaller than those from variable enzymes, but are not different from those by two-dimensional electrophoresis. The higher heterozygosity from native gel electrophoresis can be attributed to preferred use of easily assayed variable enzymes. Two-dimensional electrophoresis, on the other hand, detects more numerous proteins that may be conservative (less variable) due to physiological constraints as so specific enzymes are.

Multimeric proteins are less variable than monomeric ones (Zouros, 1976; Harris *et al.*, 1977; Ward, 1977). Likewise, structural ribosomal proteins show less variation than variable enzymes (Berger and Weber, 1974; Bucknall *et al.*, 1975). Edwards and Hopkinson (1980) and McConkey (1982) suggested that such less variable proteins are involved in macromolecular interactions, such as vital protein-protein interactions and binding between protein and lipid/carbohydrate moieties. Because of their conformational constraints, these proteins, which are parts of complex structures, are more conservative than those which are solitary or parts of simple structures. We believe that most proteins detected by two-dimensional electrophoresis are those associated with complex structures (as also suggested by McConkey).

Therefore, native gel electrophoresis of variable enzymes is advantageous for detection of genetic differences among organisms. However, amino acid or DNA sequencing may be more preferable and informative when examining such a particular and defined class of proteins. If the goal is a comprehensive and balanced view of genetic differences among organisms, then the use of two-dimensional electrophoresis seems to be advantageous, since this procedure allows examination of at least some representatives of all classes of proteins. Although the two-dimensional electrophoresis method has some difficulties and detects prevalent less variable proteins (McConkey, 1982), it is a useful method which permits us to examine many kinds of proteins at one time.

Ayala *et al.* (1974) and Avise (1975) showed that native gel electrophoresis provides a valuable tool for systematic examination of genetic divergence among closely related species or congeneric species, as well as intraspecific populations. However, because of its high sensitivity for genetic differences, this method is not useful for determining relationships at taxonomic levels above species. In this case, we must use a limited number of very specific (less variable) enzymes such as lactate dehydrogenase (Shaklee and Whitt, 1981). Based on available data (for example, see Table 1 in Aquadro and Avise, 1981) and our results, the two-dimensional electrophoresis method permits us to estimate and evaluate genetic differences at higher taxonomic levels. Furthermore, Aquadro and Avise (1981) showed that genetic distance ranks, obtained by two-dimensional electrophoresis, are highly concordant with ranks of taxonomic levels. Therefore, we suggest that this technique does provide a valuable tool for systematics.

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